Investigation of pathogenetic factors in primary Sjögren's syndrome – with special reference to an autonomic nervous system dysfunction

PhD thesis

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List of publications related to the topic of the theses

1. Gyöngyösi M, Pokorny G, Jambrik Z, Kovács L, Kovács A, Makula É, Csanády M. Cardiac manifestations in primary Sjögren's syndrome. Ann Rheum Dis 1996;55:450-454 IF.: 2.444
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1. Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease characterised by dysfunctions of the lachrymal, salivary and occasionally other exocrine glands, leading to mucosal dryness, and by a variety of extraglandular organ manifestations [1,2]. The diagnostic hallmarks of the disease are the subjective and objective signs of ocular and oral dryness termed keratoconjunctivitis sicca and xerostomia. The disease is associated with a chronic inflammatory cell infiltration of the involved organs, the predominant cell type in the infiltration being CD4-positive T-lymphocytes [3]. A B-lymphocyte hyperactivity is featured by hypergammaglobulinaemia, the presence of autoantibodies and, as an ultimate stage in a minority of the cases, the development of malignant B-cell lymphoma [4]. The syndrome can present in association with other systemic autoimmune diseases (i.e. systemic lupus erythematosus, rheumatoid arthritis [RA], systemic sclerosis, etc.), in which case it is termed secondary SS, or it can manifest as a separate entity, designated primary SS (pSS).

pSS is one of the most common systemic autoimmune diseases; its prevalence among adult females is estimated to be around 0.6% [5]. However, rather different prevalence figures have also been published, ranging from 2.7% in an elderly population [6] to 0.4% in a study focusing on autoantibody determinations [7]. This wide variety certainly reflects differences in the diagnostic criteria applied (5,7). It is about 20 times more frequent among females than among males [8]. In addition to the lachrymal and the salivary glands, dysfunctions can also occur in the exocrine glands of the upper airways, the gastrointestinal tract, the vagina and the skin, leading to bronchitis sicca, atrophic gastritis, vaginitis sicca and skin dryness, respectively. The most common extraglandular manifestation in pSS is a non-erosive polyarthritis occurring in as many as 90% of the patients [9]. Further organ involvements, such as Raynaud's phenomenon, renal tubular acidosis occurring alone or together with other signs of a chronic tubulointerstitial nephritis, small vessel vasculitis and pulmonary fibrosis are encountered in 10-30% of the patients [2,9,10]. Although the overall mortality does not seem to be higher in pSS than in the general population [11], malignant lymphoma or certain other manifestations can lead to a life-endangering state, and the morbidity due to the various clinical symptoms is almost always high [4].

The diagnosis of pSS requires the fulfilment of various diagnostic criteria. The first classification system was the Copenhagen criteria [12], while later independent groups established further systems: the San Diego [13], the Japanese [14], the Scandinavian [15] and the Greek [16] criteria. Subsequently, the European Community criteria were introduced [17] and gained wide acceptance. These criteria differed from each other in certain important points, and therefore they identified rather different patient populations which greatly hampered the evaluation of various studies of pSS patients [18]. Although the two most widely used criterion systems were recently unified as the American-European classification criteria [19], all of these diagnostic approaches require various invasive investigations (labial biopsy and sialography). The search for simple, non-invasive diagnostic methods therefore remains an important issue. Our group has made considerable efforts to assess the value of parotid gland ultrasonography and magnetic resonance imaging (MRI) in the diagnosis and follow-up of pSS patients [20,21].

With the involvement of 62 pSS patients and 63 controls, we demonstrated that the sonographic results (in particular the presence or absence of a parotid gland inhomogeneity) were in very good accordance with the parotid sialographic and scintigraphic findings and the histologic results on the minor salivary glands. The MRI studies led to similar conclusions. There was good agreement between the MRI and the ultrasonographic findings both in the pSS cases (93.2%) and in the controls (86.5%). In one pSS patient who developed parotid lymphoma, ultrasonography revealed a hypoechoic "cobblestone"-like inhomogeneous internal pattern which was coupled with an almost homogeneous MRI pattern. As similar results were later observed in further pSS patients with lymphoma, we proposed that this combination of echographic and MRI patterns may be characteristic of a lymphoma. In view of the high specificity and sensitivity of the ultrasonographic examination, and also its favourable cost, relative simplicity and wide availability, we suggested that this examination should be incorporated in the diagnostic criteria of pSS. As the MRI examination did not prove to be superior in diagnostic value to ultrasonography, we concluded that this imaging technique should be reserved for special cases.

While the classical glandular signs and symptoms of SS have been clarified more than 70 years ago [22], our knowledge of the clinical spectrum of the disease has continuously grown during the past few decades. Systematic examination of certain organs, i.e. the stomach

[23], the kidney [24,25], the peripheral nervous system [26,27] and the lung [28], has significantly widened our perspectives concerning pSS, as these investigations have revealed abnormalities in great proportions of the examined patients. Nevertheless, data on the involvement of another organ system of vital importance, the cardiovascular system, were somewhat scarce and inconclusive [29,30]). Despite the severe oral symptoms and pathological findings, little was earlier known about the oesophageal motor function either [31,32]. Accordingly, one of my activities involved participation in clinical studies of the cardiac and oesophageal functions in pSS patients.

The classical concept of the origin of the sicca symptoms in pSS was that a progressive loss of functioning glandular tissue caused by a chronic inflammation leads to irreversible end-organ damage and a consecutive cessation of secretion. However, this concept is now undergoing profound modification [18,33-35]. The symptoms of dryness and the quantitatively determined saliva and tear production may vary greatly during the course of the disease, even without treatment, and their spontaneous improvement is often observed for varying periods [36]. A marked discrepancy has been reported between the degree of exocrine insufficiency and the histologically verified intensity of the inflammatory infiltration in the salivary glands [37]. These data suggest that the sicca symptoms may partly be consequences of certain potentially reversible factors distinct from irreversible organ destruction. Oral dryness is a common side-effect of drugs with anticholinergic action. An autonomic neuropathy has been implicated in the elicitation of oral and ocular dryness in patients with diabetes mellitus [38-39]. Occasional case reports [40-43] and clinical trials involving small series of patients [44-50] have been published concerning an autonomic neuropathy in pSS. On the basis of these considerations, some investigators, including ourselves [51], have hypothesised that an impaired parasympathetic innervation of the exocrine glands, and most importantly of the salivary and the lachrymal glands, may be one such factor that contributes to the loss of secretory function [33,35,52,53].

Although studies on an autonomic nervous system dysfunction in pSS were by no means numerous in the second half of the 1990s, multiple levels of the cholinergic innervation pathway had been studied. The salivary outputs of vasoactive intestinal peptide (considered a marker of the parasympathetic innervation) and of neuropeptide Y (a marker of sympathetic innervation) were both demonstrated to be increased as compared with those in

healthy controls [54]. It was suggested that this phenomenon is associated with an altered response to stress in pSS patients. Immunohistological examinations revealed that vasoactive intestinal peptide-containing parasympathetic fibres were absent from regions of the parotid glands where inflammatory cell infiltration and acinar atrophy were severe [55]. Others reported that the expression of the β_{II} and α isoforms of protein kinase C (PKC), a crucial member of the intracellular signal transduction pathway of cholinergic neurotransmission, was deficient on the salivary gland acinar epithelial cells in pSS patients as compared with controls. In contrast with healthy individuals, the ξ and β_{II} isoforms of PKC were not detected on the myoepithelial cells in pSS patients. These findings were suggestive of a postreceptorial disturbance of the cholinergic innervation [56].

Cardiovascular autonomic neuropathy had been investigated in pSS by various study groups [44-50]; however, possibly in consequence of the differences in the methods applied, the results were not consistent. Previous studies using various cardiovascular reflex tests found signs of a parasympathetic and a sympathetic dysfunction in rather variable proportions of the patients (24 to 100% in the various tests) [44-46,49]. Non-invasive [44,49] and invasive [46] orthostatic tests revealed only minor differences between pSS patients and controls. A more up-to-date way to examine the cardiovascular autonomic nervous system function is the computerised measurement of the heart rate variability (HRV), the blood pressure variability (BPV) and the spontaneous baroreflex sensitivity (BRS). Short-term HRV examinations demonstrated lower a HRV, BPV and BRS in pSS patients than in the controls [50], while during 24-hour recordings, the HRV parameters of pSS patients did not differ significantly from those of the healthy controls [49]. In contrast, only one article mentioned gastric motility in pSS [57], and no clinical trial was published on urogenital involvement. Thus, results suggestive of certain disturbances of the cholinergic neurotransmission at both pre-receptorial and post-receptorial levels were obtained.

An important finding of further examinations of this putative autonomic dysfunction was that autoantibodies that react with the muscarinic acetylcholine receptor (mAchR) are detectable in the sera of pSS patients. Two independent working groups have demonstrated that the immunoglobulin-G (IgG) fraction of the sera of pSS patients binds to purified and solubilised mAchRs of the mouse and the rat submandibular salivary gland, and behaves as a non-competitive agonist of these receptors in a receptor binding assay [58,59]. These

observations were based on the following findings: While non-obese diabetic (NOD) mice develop a lymphocytic infiltration in the exocrine glands and a sicca syndrome similar to that seen in human pSS patients, Igμ-null transgenic NOD mice, which are unable to produce antibodies do not have an exocrine insufficiency, despite the fact that they develop lymphocytic infiltrations in the salivary glands similar to those in the wild-type mice. The transfer of pSS IgG to Igμ-null transgenic mice led to the loss of saliva production in these mice, thereby proving that certain antibodies are responsible for the elicitation of the exocrine dysfunction in this murine model of pSS. The above action of pSS IgG was completely antagonised with mAchR antagonist agents, demonstrating that an IgG fraction binding to mAchR is essential for induction of the sicca symptoms [59,60].

Subsequently, the presence of antibodies binding to the human m3AchR, and specifically to a synthetic 25mer peptide presumed to correspond to the 2nd extracellular loop of the human m3AchR, was also verified in pSS patients [61]. However, in a recent study, the authors could not confirm the presence of antibodies reactive with the above synthetic peptide. Moreover, it was demonstrated that this sequence, albeit having a 70% homology with the human m3AchR section, actually corresponds to the 2nd extracellular loop of the human m4AchR [62]. Nevertheless, the authors also failed to detect a reaction between pSS sera and the correct peptide fragment of the acetylcholine-binding region of the m3AchR, nor with two further epitopes on the m3AchR. Therefore, no conclusive demonstration of the presence of antibodies reacting with the human m3AchR, considered a crucial factor in the pathogenesis of pSS, has been achieved.

Inspired by these novel findings, a major part of my research work was an investigation of the autonomic nervous system function in pSS patients by means of clinical tests, and an attempt to verify the presence of anti-human mAchR autoantibodies in such patients. First, in order to investigate the response of the parasympathetic nervous system to certain agents directly, we designed an experimental model involving the cutaneous microcirculation to test whether there are differences between pSS patients and healthy controls in the reaction to the local administration of a muscarinic cholinergic agent. This *in vivo* functional test was also suitable to elucidate whether a putative autonomic disorder exists only in the exocrine glands, or is a general phenomenon in pSS. A cholinergic vasodilatory mechanism of the human skin blood vessels has long been verified [63-65]. Since this is

similar in many respects to the cholinergic innervation of the salivary glands [64], we decided to measure the cutaneous vascular response to the local administration of a cholinergic agonist, and to use this experimental setting to test the hypothesis that a cholinergic dysfunction exists in pSS patients. Second, as the m3AchR is the functionally predominant receptor subtype not only in the salivary gland, but also in the gastrointestinal and urogenital systems [66,67], we hypothesised that anti-m3AchR antibodies also bind to receptors located in these organs and cause an autonomic dysfunction. Therefore, with clinical tests, we investigated whether signs of an autonomic dysfunction can be detected in the gastrointestinal and urogenital systems. Furthermore, the clinical importance of a cardiovascular autonomic neuropathy and the inconsistencies in the results of the previous studies in this field prompted us to attempt to clarify the occurrence and severity of a cardiovascular autonomic neuropathy by using both the standard cardiovascular reflex tests and the more sensitive and up-to-date HRV examinations. Finally, as the presence of autoantibodies to the human m3AchR had not been convincingly confirmed, my aim was to develop an experimental system with which to examine whether anti-mAchR antibodies can be detected in the sera of pSS patients.

In conclusion, the aim of these trials was to gain more insight into the mechanisms of a putative autonomic nervous system dysfunction in pSS patients.

2. Aims

On this basis, my aims were to investigate the following features:

1. The presence, occurrence and severity of echocardiographically detectable cardiac abnormalities and an oesophageal dysfunction in pSS patients.
2. The responsiveness of pSS patients to cholinergic stimuli in a microcirculatory model.
3. The frequency and characteristics of an autonomic nervous system dysfunction involving the cardiovascular, gastrointestinal and urinary systems in pSS patients.
4. The development of a diagnostic system for the detection of antibodies reacting with human mAchRs and the examination of whether such antibodies are present in the sera of pSS patients.

3. Methods and Results

3.1. Investigation of cardiac and oesophageal involvement in pSS

3.1. 1. Cardiac manifestations

Sixty-four pSS patients (62 female and 2 male, mean age: 59 [SD 12] years, European Community criteria [17]) were examined with standard M-mode, two-dimensional and colour Doppler echocardiography. Twenty-one age-matched healthy women served as controls.

The most common abnormal finding in the pSS patients was an impaired left ventricular diastolic function, which was revealed in 50% of the 42 patients who could be evaluated in this respect. An echogenic pericardium was demonstrated in 21 patients (33%), while acute exsudative pericarditis was detected in only one patient. The pulmonary pressure was significantly greater in the patients (31 [SD 8] mm Hg) than in the controls (24 [SD 7] mm Hg) ($p<0.05$).

3.1.2. Oesophageal involvement

Twenty-five pSS patients (22 female and 3 male, mean age: 55 [range: 31-75] years, European Community criteria [17]) were examined with standard, water-perfusion oesophageal manometry. The amplitude, duration and propagation velocity of the contractions in the oesophageal body were measured, together with an assessment of the function of the lower and upper oesophageal sphincters and the pharyngeal muscles. The results were compared with those on 42 healthy controls and were also correlated with the patient characteristics and with symptoms related to oral dryness or swallowing disorders.

In the oesophageal body, we observed an increased duration of peristaltic contractions, a decreased propagation velocity and a higher rate of simultaneous contractions for both dry and wet swallows. The mean lower sphincter pressure was significantly lower and the mean relaxation times were significantly longer in the pSS patients as compared with the controls. The predominant motor abnormality appeared to be a decreased oesophageal body peristaltic velocity, which correlated with the decrease in both stimulated and unstimulated whole saliva production, but with none of the other patient characteristics.

3.2. Responsiveness of pSS patients to cholinergic stimuli

3.2.1. Patients and controls

Twenty-two primary SS patients (20 female and 2 male) were enrolled in the study. All were diagnosed as having SS following the European Community criteria [17]. The average age of the patients was 50.6 ± 13.2 years (33-60). In order to exclude other factors that might possibly influence the microcirculatory physiology, SS patients older than 60 years, those who had hypertension or clinical evidence of arteriosclerosis (coronary heart disease, arteriosclerotic cerebral disease or arteriosclerosis in other organs) or peripheral neuropathy, and those who regularly took beta-adrenergic blockers, calcium-channel blockers, pentoxyphylline, or other drugs with vasodilatory or anticholinergic properties, were regarded as not eligible for the study. For the same reason, the use of non-steroidal anti-inflammatory drugs was suspended at least 5 days before the examinations. The patients were asked to refrain from smoking or the drinking of coffee on the day of the examination.

Twelve healthy, age- and sex-matched individuals were examined as controls. None of these subjects had any known illness, or were on any regular medication.

3.2.2. Examination of the cutaneous vascular response

During examinations, the patients and the healthy controls were in the supine position. The skin blood flow (SBF) was measured with a laser-Doppler flow-meter (Penflux, Perimed) attached to the skin on the flexor surface of the right forearm, and blood flow values were expressed in arbitrary units [68]. Three ECG electrodes were attached to the chest and the heart rate (HR) was monitored continuously, as was the blood pressure (BP) by means of a photoplethysmographic BP monitor (Finapres 2300, Ohmeda) attached to a finger of the right hand (beat to beat registration). The subjects were asked to lie at rest with their eyes closed. The baseline SBF was recorded for 5 minutes; the blood flow was stable by the end of this period and the SBF value measured at 5 minutes was defined as SBF_{basal}. 0.1 ml of carbachol (Miostat, Alcon, USA), a muscarinic receptor agonist, was then injected intracutaneously into the forearm skin. As control, almost simultaneously, 0.1 ml of 0.9% saline solution was injected similarly into the forearm skin at approximately 10 cm from the other injection site. SBF was measured simultaneously at the two injection sites for another 10 minutes and the highest deviations from the baseline flow values were designated SBF_{final}.

All recorded data (HR, systolic and diastolic arterial BP [SBP and DBP], and SBF) were stored in a computer database and were analysed by means of self-developed software. The change in SBF in response to the injection of carbachol (dSBF) was calculated via the following formula:

$$dSBF = \frac{SBF_{final,carbachol}/SBF_{basal,carbachol}}{SBF_{final,saline}/SBF_{basal,saline}}$$

Thus, dSBF is the ratio of the SBF values measured after and before the injection of carbachol divided by the corresponding ratio for the control solution of physiological saline. This calculation allowed elimination of the absolute flow values and also elimination of possible non-specific microcirculatory effects of the intracutaneous injection. In every subject, the mean values of the R-R intervals on the ECG, and the systolic, diastolic and mean arterial

pressures were also recorded. The cutaneous vascular resistance (CVR) was calculated by dividing the mean arterial BP by SBF, while dCVR was calculated with a formula analogous to that for dSBF.

3.2.3. Results

Table I reports the average values of the most important haemodynamic variables in the two groups recorded during the examinations of the cutaneous microcirculation. In the event of a positive vascular reaction, the blood flow started to increase within 30 seconds after the injection of carbachol and reached a new steady state approximately 4-7 minutes later, then remaining unchanged until the end of the examination. In the controls, the average dSBF was 3.30 ± 1.79, which was significantly higher than the average dSBF in SS patients (2.07 ± 1.12) (p=0.019) *(Figure 1).* In the controls, the dSBF values reflected a 1.66-7.6-fold increase in microcirculatory blood flow after the injection of carbachol. However, in a relatively high proportion of the SS patients, the reaction to the administration of carbachol was small or virtually absent, while in other patients a marked vasodilation was observed. We defined a positive microvascular reaction to carbachol in the pSS patients as a dSBF value higher than

Table I. A comparison of certain haemodynamic variables in primary Sjögren's syndrome (pSS) patients and controls recorded during the blood flow examinations.

Group	$SBF_{final,carba\text{-}chol}$ / $SBF_{basal,carba\text{-}chol}$	$SBF_{final,saline}$ / $SBF_{basal,saline}$	dSBF	dCVR	Mean RRI (msec)	Mean SBP (mmHg)	Mean MBP (mmHg)	Mean DBP (mmHg)
pSS patients	1.89 ± 1.05 *	0.94 ± 0.29	**2.07± 1.12¶**	0.64± 0.32•	827.9± 109.6	132.4± 22.0	95.9± 16.5	76.3± 14.9
Controls	2.72 ± 1.27	0.86 ± 0.19	**3.30± 1.79**	0.38± 0.17	797.0± 117.3	128.6± 14.9	93.8± 9.8	74.9± 7.5

Numbers indicate means ± SD. SBF: skin blood flow; CVR: cutaneous vascular resistance; RRI: RR-interval on ECG; SBP: systolic arterial blood pressure; MBP: mean arterial blood pressure; DBP: diastolic arterial blood pressure.

SBF and CVR values are in arbitrary units. For the calculation of dSBF and dCVR, see the text (*Section 3.2.2*).

* p = 0.048; ¶p = 0.019; • p = 0.013

the smallest dSBF value in the group of healthy controls. Following this definition, exactly half of the SS patients (11 of 22) could be considered to be non-responders, i.e. producing a less than 1.66-fold increase in the microcirculatory blood flow, while the other half of the patients were regarded as responders to carbachol on the basis of the pronounced vasodilatation (a more than 1.66-fold increase in SBF).

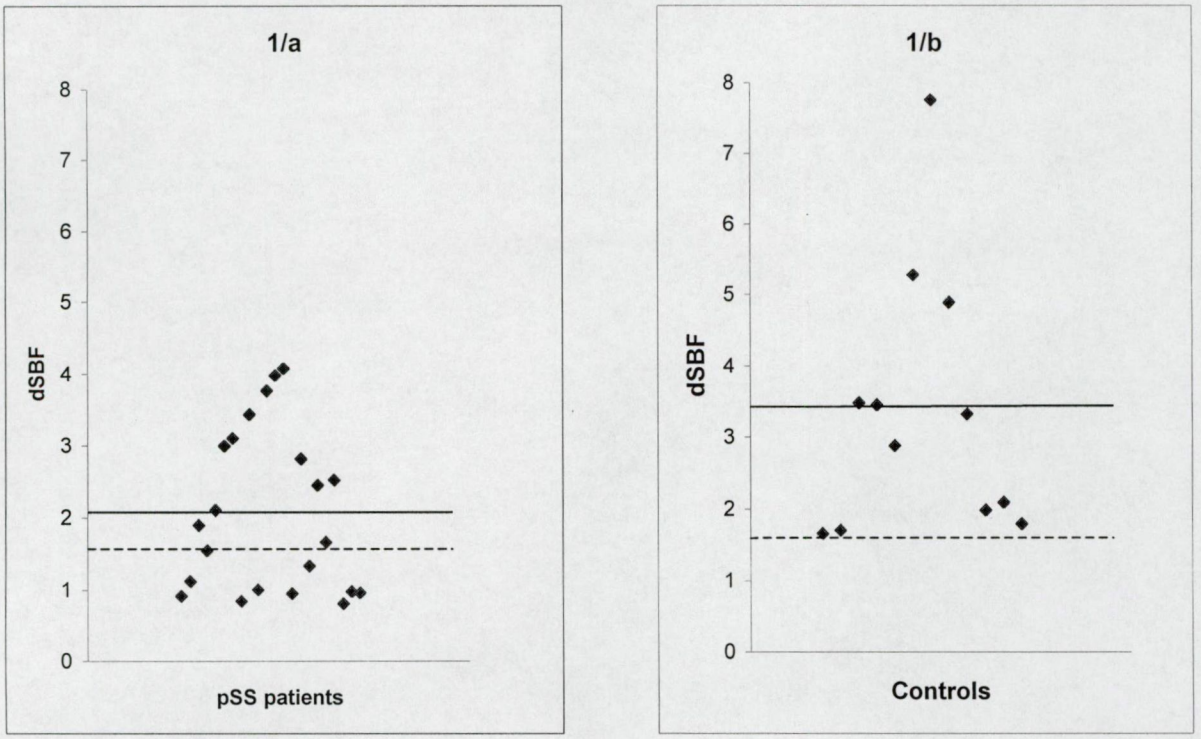


Figure 1. The distribution of dSBF values in primary Sjögren's syndrome (pSS) patients and healthy controls.

dSBF: change in skin blood flow in response to the injection of carbachol. The continuous lines indicate means. Values above the dashed lines indicate a positive response to carbachol.

We examined whether there was any difference in certain demographic or clinical characteristics between the pSS patients defined as non-responders or as responders to carbachol (*Table II*). No statistically significant difference was observed with respect to any of the following parameters: the age of the patients; the time since the appearance of the first symptom of pSS; the occurrence of any of the main organ manifestations (but it is noteworthy that all the examined organ involvements occurred more frequently in the responder patients with the exception of pulmonary fibrosis, which was found in one patient in each of the

subgroups); the average values of tear and saliva production; the proportions of patients treated with non-steroidal anti-inflammatory drugs, hydroxychloroquine or corticosteroids; and the frequencies of anti-SSA, anti-SSB antibody, rheumatoid factor (RF) or antinuclear antibody (ANA) positivity.

Table II. Demographic characteristics and occurrences of certain organ manifestations of primary Sjögren's syndrome (pSS) in carbachol-responder and non-responder pSS patients

	Responders (n=11)	**Non-responders (n=11)**
Average age (years)	51.0	50.3
Average time since first symptom (years)	11.8	11.2
Articular involvement	9	8
Renal involvement	3	1
Bronchitis sicca	3	1
Pulmonary fibrosis	1	1
Raynaud's phenomenon	5	1
Skin vasculitis	2	1
MALT lymphoma in parotid gland	2	0

For the organ manifestations, the numbers indicate the number of involved patients. Articular involvement: arthritis or arthralgia not due to degenerative joint disease. Renal involvement: renal tubular acidosis or biopsy-proved tubulointerstitial nephritis. No significant difference was found between the two subgroups. MALT: mucosa-associated lymphoid tissue.

3.3. Clinical trials for the examination of the spectrum of autonomic nervous system dysfunction in pSS

3.3.1. Study patients

From the cohort of pSS patients followed up at our Department, all who did not have a disease that may cause an autonomic neuropathy (e.g. diabetes mellitus or chronic renal failure), and were younger than 75 years were enrolled in this study (51 pSS patients [48 women], average age: 53 [range 31-71] years; American-European classification criteria [19]). A questionnaire relating to symptoms which may potentially be caused by an autonomic dysfunction was completed by every patient. An answer was considered positive when no other medical condition potentially attributable to the elicitation of the symptom was

present in the given patient. For the clinical tests, further exclusion criteria were defined; the numbers of patients participating in the individual clinical examinations therefore varied.

3.3.2. Methods - Examination of heart rate and blood pressure variability

The examination of HRV and BPV is a sensitive method for assessment of autonomic neural effects on the cardiovascular system [69]. It is also suitable for measurement of the spontaneous BRS. HR and BP were recorded continuously for a period of 10 minutes. with a Marquette bedside ECG monitor, and a beat-to-beat plethysmographic BP monitor (Finapres 2300, Ohmeda, St. Louis, MI, U.S.A.), respectively. Signals were recorded on-line and digitalised with a Dataq/Windaq system. The data were analysed off-line, using custom software (WinCPRS). The following standard HRV and BPV indices were calculated:

1. Time domain analysis yields descriptive statistical data on the variability of the cycle length (R-R wave interval; RRI) and BP values registered during the 10-minute examination period: minimum, maximum and mean RRI; SDRR (standard deviation of the mean RRI); rMSSD (the square root of the average of the squared differences between each consecutive pair of RRIs); pNN50 (the percentage of intervals that differed from the adjacent interval by > 50 msec); and the minimum, maximum and mean systolic BP (SBP) (69).

2. Frequency domain analysis involves power spectral analysis of the effects modulating the oscillations in the RRIs and SBP. With a fast Fourier transformation of the RRI and SBP values, 2 frequency bands can be produced: low-frequency (LF: 0.05-0.15 Hz) and high-frequency (HF: 0.15-0.5 Hz) components.

3. As the vagus nerve is the efferent pathway of the baroreflex circuit, the measurement of BRS provides precise data on the parasympathetic innervation of the heart (70). Spontaneous BRS can be assessed at rest during the simultaneous registration of RRI and SBP values, as with an intact baroreflex function every spontaneous rise or fall in BP is immediately followed by a reduction or elevation of HR. Spontaneous sequences were therefore searched for. Spontaneous sequences were defined as three or more consecutive cycles of either a SBP elevation (up-sequences) or fall (down-sequences) coupled with RRI changes in the same direction. For all of these sequences, the slope of the function of delta RRI-and delta-SBP was calculated and averaged separately for up- and down-sequences.

As the HRV indices display great variability in the healthy population [71], we compared our results on pSS patients with our previous results obtained by the systematic examination of a large healthy population [71]. Thus, the results on each pSS patient were expressed as percentile values of those for the age- and sex-matched group of healthy subjects. Three patients were ineligible for the HRV/BPV examinations because of a previous myocardial infarction, a grade III mitral valve insufficiency or frequent extrasystolia (one patient each). Since the control group comprised persons with a maximum age of 60 years, only pSS patients aged 60 years or below (39 patients, 37 female) could eventually be evaluated and compared with a cohort of 559 healthy subjects [71]. The evaluation of BRS is feasible only when sufficient numbers of up-BRS and down-BRS sequences are available for the statistical analysis. Further patients therefore had to be excluded from the evaluation process; finally, up-BRS values could be calculated in 30 patients and down-BRS values in 27 patients.

3.3.3. Methods - Cardiovascular reflex tests

Another approach with which to assess the cardiovascular autonomic nervous function is the performance of cardiovascular reflex tests. Five simple, fast and well-reproducible tests were carried out following the standard methods of Ewing *et al.* [72]: 1. HR changes in response to deep breathing. 2. HR changes in response to the Valsalva manoeuvre. 3. HR changes in response to standing up (30/15 ratio). 4. SBP changes in response to standing up. 5. DBP changes in response to a sustained handgrip. In every test, normal values were scored 0, borderline values 1 and abnormal values 2. The sum of the scores gave the total autonomic neuropathy score [72]. A score of 0-1 was taken as normal, 2-3 as borderline and >3 as abnormal [72]. Of the original 51 patients, 9 were ineligible for the reflex tests because they regularly took beta-adrenergic blockers or vasodilating drugs, and the above-mentioned 3 patients with various organic cardiac abnormalities were also excluded. Thus, the tests were performed on 39 patients (37 female) and the results were compared with those on 39 age- and sex-matched healthy controls.

3.3.4. Methods - Examination of gastric emptying

The gastrointestinal autonomic nervous function was examined by assessment of the gastric motility with gastric emptying scintigraphy. Patients with an organic upper gastrointestinal disease that may influence the results of the scintigraphic studies were regarded as ineligible for this examination. One patient with previous antral surgical polypectomy and another patient with pernicious anaemia were therefore excluded. Of the remaining subjects, 30 consecutive patients (27 female) participated in this study. The examination was performed in the morning after an overnight fast. The patients ingested a radiolabelled meal (2 hard-boiled eggs labelled with 20 MBq 99^{m}Tc-human serum albumin macroaggregate + one bread roll and 200 ml water). A dynamic scintigraphic study was performed about the gastric region. Digital images were taken at a frequency of 1 minute/frame for 2 hours. As a parameter of gastric emptying, the emptying half-time ($t_{1/2}$), i.e. the time until the radioactivity in the stomach had decreased to half the initial value, was determined.

During the validation process of this procedure, the cut-off value for an abnormal $t_{1/2}$ was determined as the average + 1 SD of the $t_{1/2}$ values for 7 healthy individuals (6 women) with an average age similar to that of the pSS patients. Thus, a $t_{1/2}$ value >74 minutes was considered abnormal.

3.3.5. Methods - Urodynamic examinations

For assessment of the autonomic neural effects on the urinary tract, standard urodynamic examinations were performed. After detailed diagnostic investigations of the pelvic organs, 5 women with cystocele and 3 men with bilateral prostate hypertrophy proved to be ineligible for the urodynamic examinations. Six patients refused to participate, and finally 16 consecutive patients (all female) underwent the investigation. Uroflow measurements and cystometric examinations were carried out in a manner completely identical to the routine diagnostic examinations. An overall evaluation of all the clinical data and the urodynamic charts was made following the international guidelines for diagnostic urodynamic examinations [73]. As an autonomic nervous system dysfunction leads to an altered detrusor muscle tone or contractility, the following parameters were considered for the purposes of statistical analysis: maximum cystometric bladder capacity (normal: 320-590 ml),

peak detrusor pressure (i.e. the difference between the peak intravesical pressure and the intra-abdominal pressure; normal: 35-60 cmH_2O), and the maximum urinary flow rate (normal: 15-36 ml/sec). The normal values we applied are standard values established for adult females [73].

3.3.6. Results - HRV and BPV examinations

The results of the time domain and frequency domain analyses of HRV and BPV in the pSS patients are depicted in *Figures 2* and *3*. For the purpose of comparison with the healthy controls, the 37 participating pSS patients were allocated into four subgroups according to age and sex: women aged 30-39, 40-49 or 50-59 and men aged 50-59. For each of the test parameters, we defined the percentile values in the appropriate age and gender subgroup of healthy controls for the results on every patient. The distribution of the percentile values for the pSS patients is represented in *Figures 2* and *3*. It can be seen that the mean RRI and SBP values were distributed relatively evenly in the patients, similarly to their distribution in the healthy population ($p>0.05$). In contrast, the SDRR, pNN50, rMSSD, LF-RRI, HF-RRI and LF-SBP percentile values were clustered in the lowest percentile range, i.e. a majority of the pSS patients exhibited parameters that were below the 20^{th} percentile in the corresponding age- and sex-matched healthy population. In contrast, the HF-SBP values peaked in the high percentile range. The distributions of all the above parameters were significantly different from the normal distribution ($p<0.001$). *Figure 4* shows that in almost 50% of the patients the up-BRS and down-BRS values were below the 20^{th} percentile range, while the results on the remainder of the patients were distributed relatively evenly between the other percentile ranges (a significant difference from the normal distribution; $p<0.05$). In summary, pSS patients have HR and BP values similar to those in the healthy population, but the variability in both HR and BP is restricted and BRS is decreased.

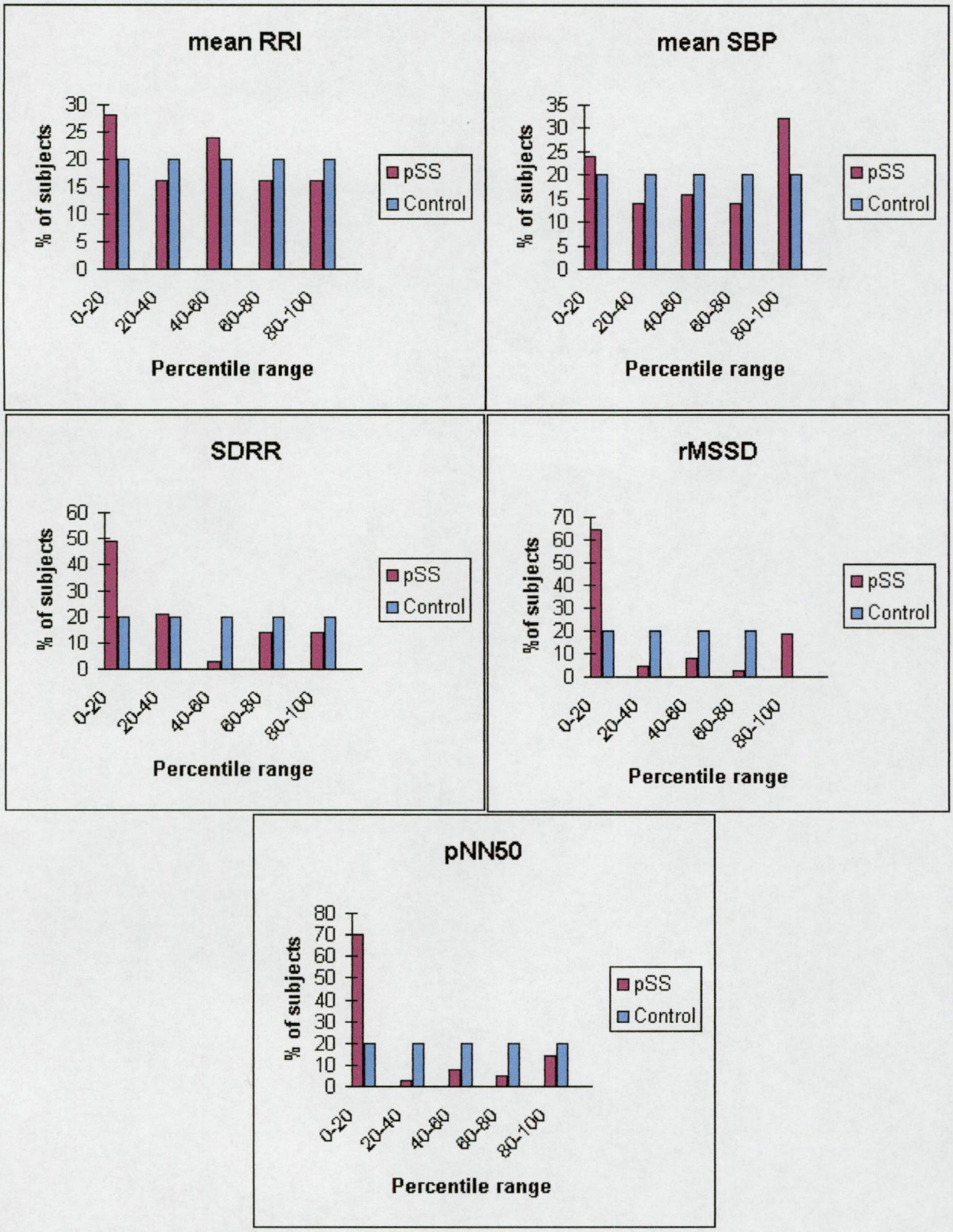


Figure 2. Results of time domain analysis of heart rate and blood pressure variability of 39 primary Sjögren's syndrome (pSS) patients.

The results are represented as percentile values of those for a group of age- and sex-matched healthy individuals. As an illustration, the percentile distribution of the healthy control population is also presented. The distributions of the patients in the various percentile ranges are significantly different from the expected distribution for SDRR, pNN50 and rMSSD ($p<0.001$). For the abbreviations and more details, see subsection 3.3.2.

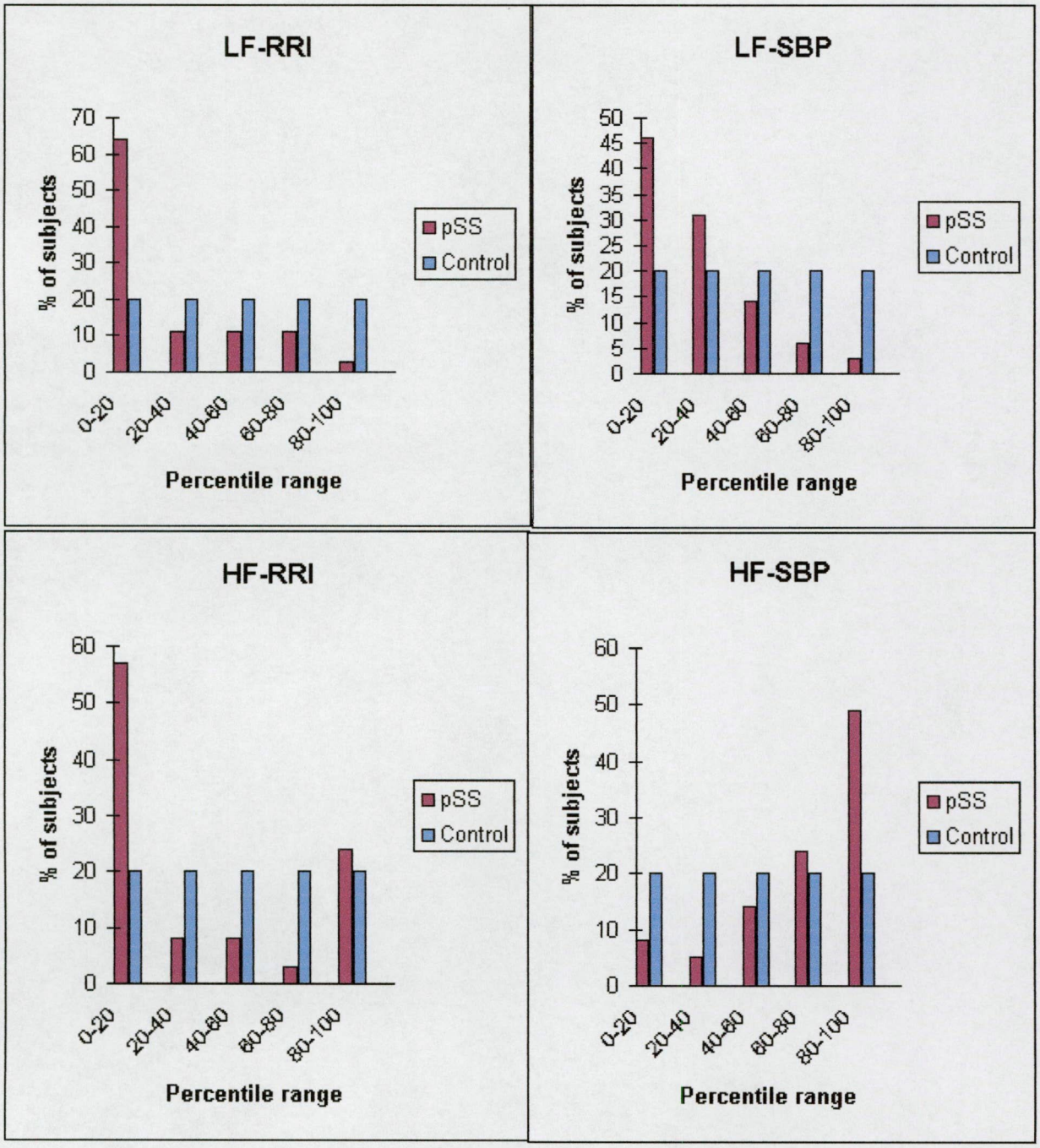


Figure 3. Results of frequency domain analysis of heart rate and blood pressure variability of 39 primary Sjögren's syndrome (pSS) patients.

The results are represented as percentile values of those for a group of age- and sex-matched healthy individuals. As an illustration, the percentile distribution of the healthy control population is also presented The distributions of the patients in the various percentile ranges are significantly different from the expected distribution for each of the parameters ($p<0.001$). For the abbreviations and more details, see subsection 3.3.2

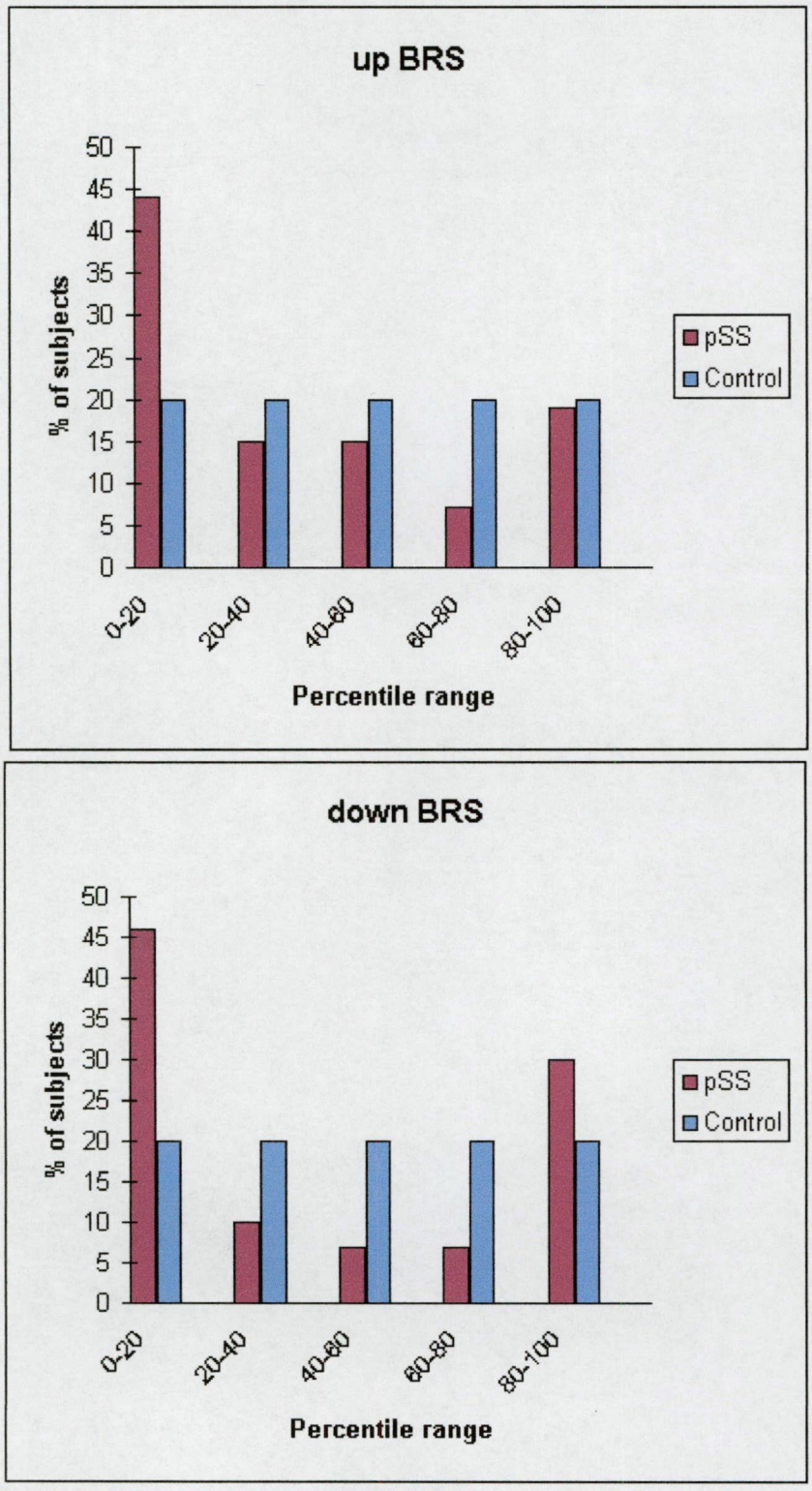


Figure 4. Results of baroreflex sensitivity (BRS) examinations of 39 primary Sjögren's syndrome (pSS) patients.

The distributions of the up-BRS (30 patients) and the down-BRS (27 patients) values of the patients are presented as percentile values of those for a group of age- and sex-matched healthy individuals. As an illustration, the percentile distribution of the healthy control population is also shown. The distributions of the patients in the various percentile ranges are significantly different from the expected distribution for both the up-BRS ($p<0.001$) and the down-BRS ($p<0.05$) values. For more details, see subsection 3.3.2.

Results - Cardiovascular reflex tests

The proportions of pSS patients who exhibited normal, borderline or abnormal results in the individual reflex tests, and their comparison with the results on healthy controls, are to be seen in *Figure 5*. In every test, the higher percentage of pSS patients than that of the controls demonstrated an abnormal result (in the statistical analysis, borderline and abnormal results were both considered abnormal), and this difference was statistically significant in the following tests: the HR responses to deep breathing and standing up, and the DBP change in response to a sustained handgrip ($p<0.05$). When the average values were compared between the two groups (*Table IV*), the pSS patients exhibited significantly lower median values in the

Table IV. Median values of Ewing's 5 cardiovascular reflex test results in primary Sjögren's syndrome (pSS) patients and controls

Test				pSS patients (median)	Controls (median)	p value
	Normal	Border-line	Abnor-mal			
1. Heart rate change in response to deep breathing	≥15	11-14	≤10	14	21	<0.05
2. Heart rate change in response to the Valsalva manoeuvre	≥1.21	1.11-1.20	≤1.10	1.48	1.64	>0.05
3. Heart rate change in response to standing up (30:15 ratio)	≥1.04	1.01-1.03	≤1.00	1	1.12	<0.001
4. Systolic blood pressure fall in response to standing up (orthostatic test)	<10	10-29	≥30	10	7	>0.05
5. Diastolic blood pressure elevation in response to a sustained handgrip	>16	11-15	≤10	16	25	<0.001

The validated normal values of the tests (72) are also presented. 1. Difference in maximum and minimum heart rate (HR) during a period of 6 deep inspirations and expirations (beats/min). 2. Ratio of the longest RR-interval registered within 15 seconds after a standardised Valsalva manoeuvre and the shortest RR-interval during the manoeuvre (Valsalva ratio). 3. Ratio of HRs measured 15 and 30 seconds after standing up from a supine position (beats/min). 4. Drop in systolic blood pressure after standing up from a supine position (mm Hg). 5. Difference between the maximum diastolic blood pressure measured within a period of 3 minutes during compression of the pressure gauge of a blood pressure meter and baseline diastolic blood pressure value (mm Hg). n=39 for both groups, Mann-Whitney U-test.

Figure 5/a

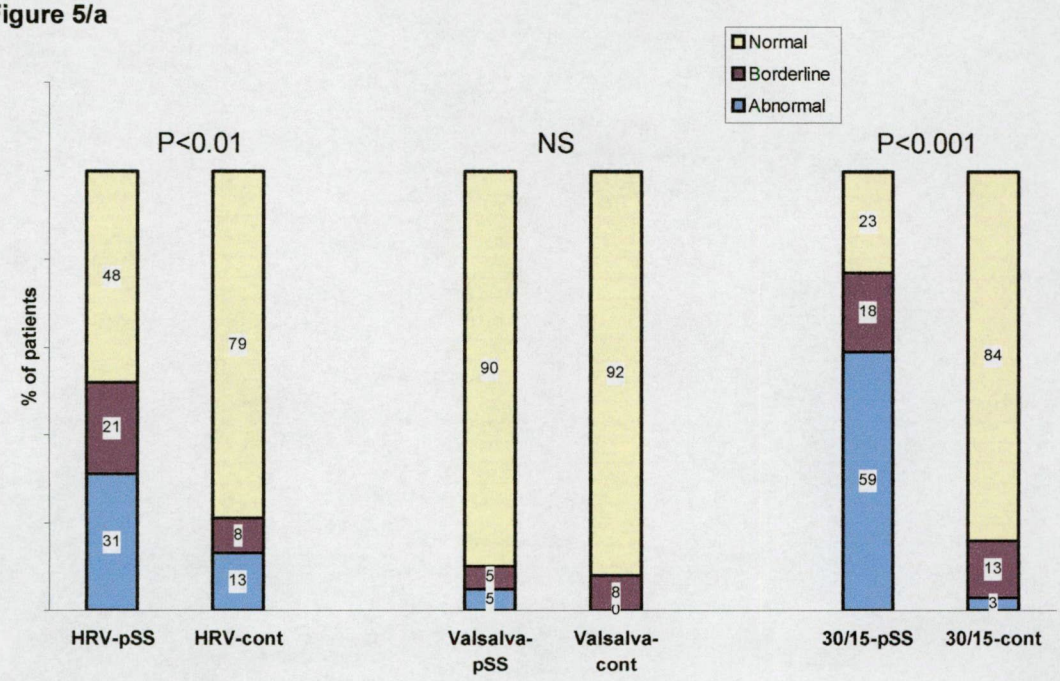


Figure 5/b

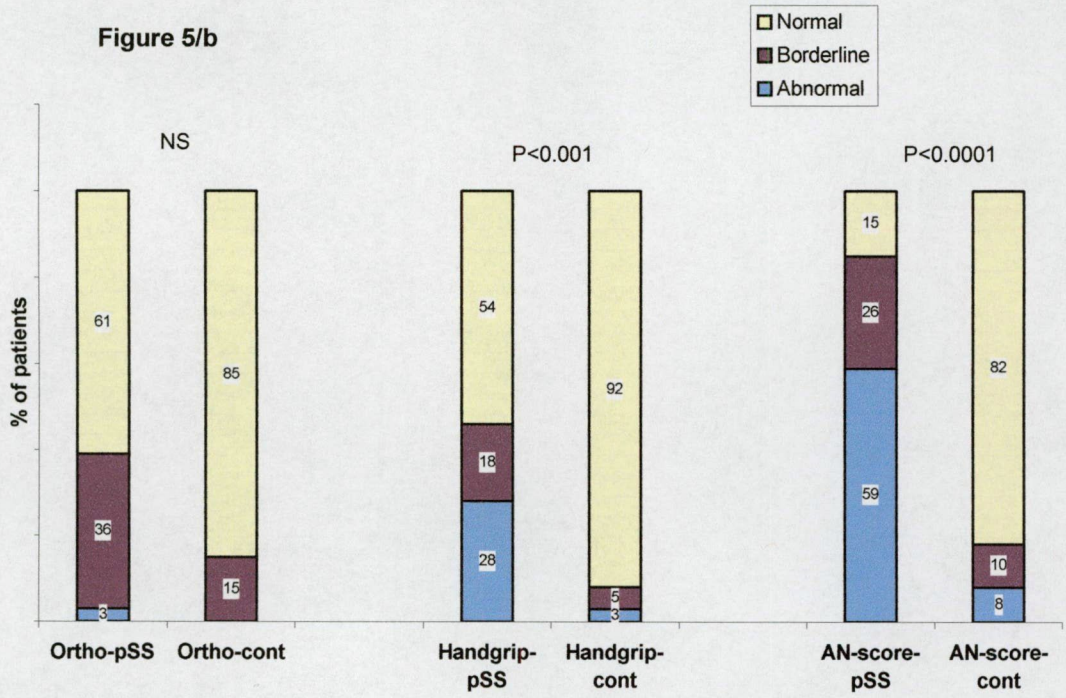


Figure 5. *Distributions of results of cardiovascular reflex tests in primary Sjögren's syndrome (pSS) patients and controls.*

Figure 5/a: tests mainly relating to parasympathetic function: heart rate changes in response to deep breathing (HRV), the Valsalva manoeuvre, and standing up (30/15). *Figure 5/b:* tests mainly relating to sympathetic function: systolic blood pressure changes in response to standing up (orthostatic challenge: ortho) and diastolic blood pressure changes in response to a sustained handgrip (handgrip). Total autonomic neuropathy score (AN score) results are also presented. Cont: control, NS: non-significant difference.

tests of the HR response to standing up ($p<0.05$) and deep breathing ($p<0.001$), and the DBP change in response to a sustained handgrip ($p<0.001$), while in the two remaining tests, the average values of the two groups were not significantly different. Sixteen patients gave abnormal and 17 borderline autonomic neuropathy scores (total: 33; 85%), while 1 control patient had abnormal and 2 had borderline autonomic neuropathy scores (total: 3; 18%; $p<0.0001$). The median autonomic neuropathy score in the patient group was 3 (range 1-8), while that in the control group was 0 (range 0-5; $p<0.0001$).

3.3.8. Results - Gastric emptying scintigraphy

The gastric emptying was significantly slower in the pSS patients than in the healthy controls. The average (± SD) $t_{1/2}$ of gastric emptying was 94 ± 35.9 minutes in the patients and 59.6 ± 16.7 minutes in the controls ($p<0.05$). Twenty-one of the 30 pSS patients (70%) yielded an abnormal gastric emptying $t_{1/2}$; moreover, in 9 of them, a markedly elevated $t_{1/2}$ (more than 120 minutes) was observed. Representative images of the normal gastric emptying of a healthy control subject and the prolonged gastric emptying of a pSS patient are shown in *Figure 6*.

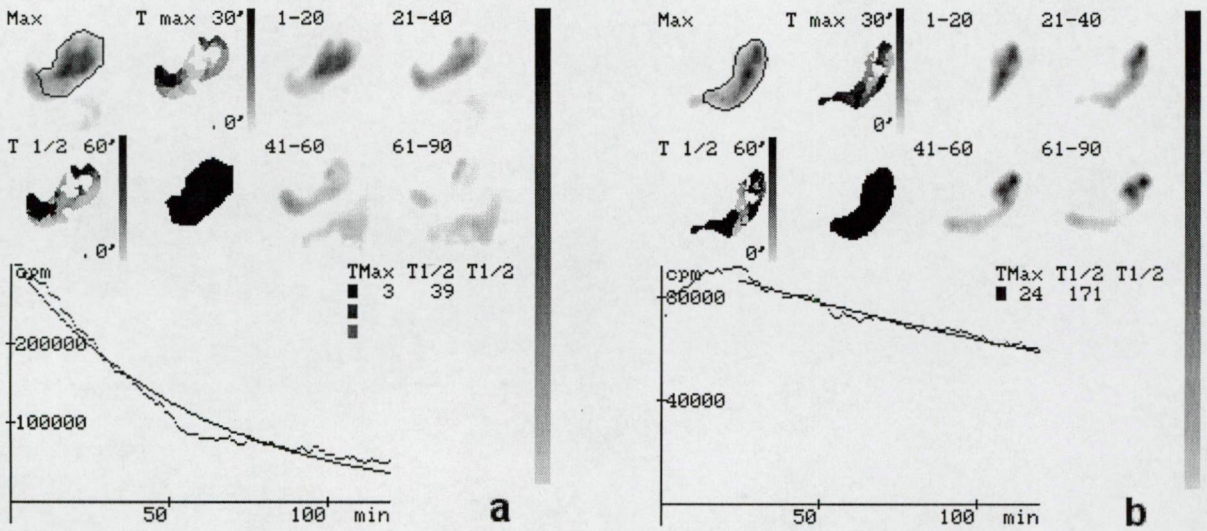


Figure 6. Radionuclide gastric emptying study of a healthy control subject (Figure 6/a), and prolonged gastric emptying of a pSS patient (Figure 6/b).

Gastric emptying $t_{1/2}$ was 39 minutes in Figure 6/a, and 171 minutes in Figure 6/b. The graphs show the radioactivity (cpm) recorded of the region of the stomach as a function of time (minutes).

3.3.9. Results - Urodynamic examinations

We detected an abnormally high bladder capacity in 9 (56%) of the 16 examined pSS patients, and an abnormally low capacity in 1 patient (mean: 553 ml, SD: 152 ml). The peak detrusor pressure was lower than normal in 6 patients (38%), while a decreased maximum uroflow value was found in 5 (31%). At least 1 of the latter 2 tests was abnormal in 9 patients; thus, 56% of the patients exhibited some sign of a decreased detrusor muscle contractility. On the other hand, for each of the above parameters, one patient demonstrated an abnormally high value (different patients in the two tests). Examples of cystometric charts demonstrating normal conditions, an abnormally high bladder capacity, and a decreased peak detrusor muscle pressure are presented in *Figure 7.*

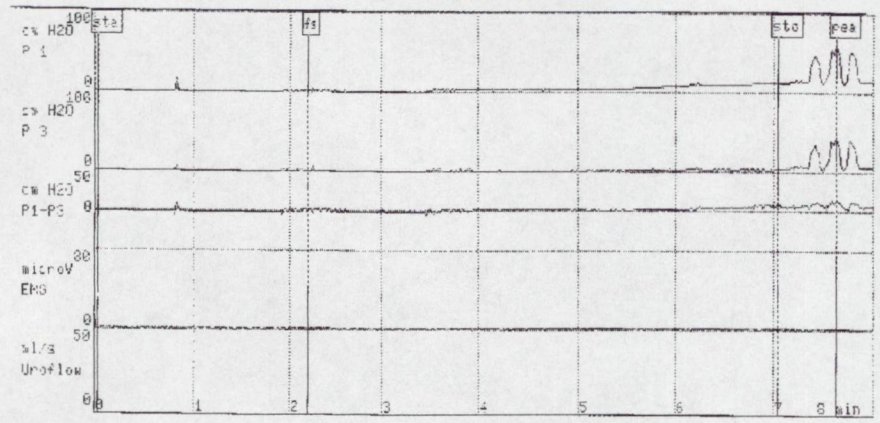
3.3.10. Results – Symptoms and correlations between test results and patient characteristics

The number of patients who experienced particular symptoms potentially attributable to an autonomic dysfunction are demonstrated in *Table V.*

Table V. Percentages of 51 pSS patients complaining of symptoms possibly attributable to an autonomic dysfunction.

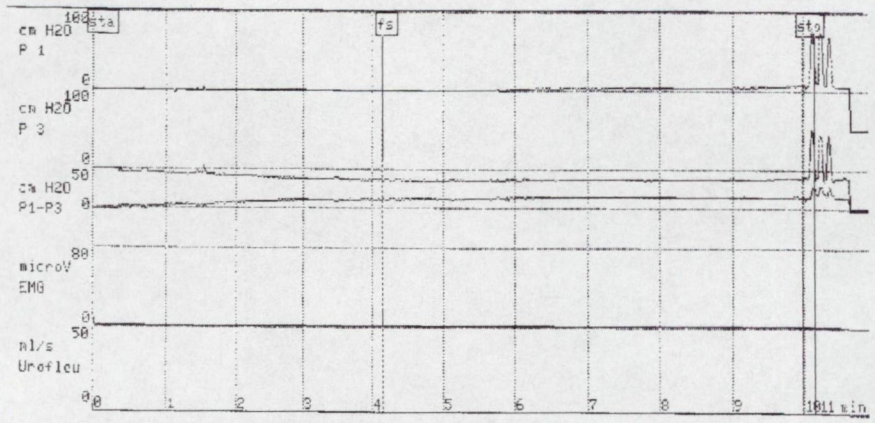
Complaint	**No.(%)**
Palpitations	9(16)
Orthostatic hypotension	3(6)
(Pre)syncope	1(2)
Postprandial fullness	6(12)
Vomiting/nausea after eating	1(2)
Bloating	4(8)
Diarrhoea	3(6)
Urge incontinence	4(8)
Stress incontinence	8(16)
Difficulty in starting voiding	1(2)

A



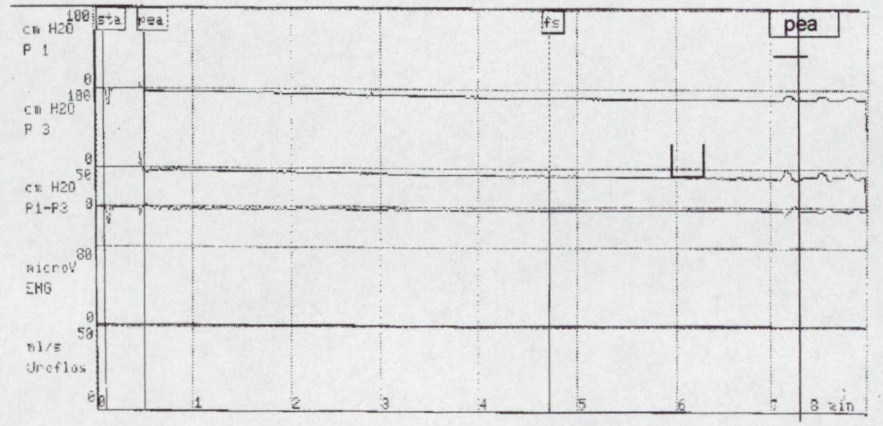
Bladder volume:
502 ml
Peak P1-P3:
17 cmH_2O

B



Bladder volume:
687 ml (filling time: 10'30")
Peak P1-P3:
33 cmH_2O

C



Bladder volume:
351 ml
Peak P1-P3:
5 cmH_2O

Figure 7. Cystometric charts of 3 primary Sjögren'ssyndrome (pSS) patients.

Panel A: normal conditions. *Panel B:* increased cystometric bladder capacity. *Panel C:* decreased detrusor muscle contractile pressure. The charts represent pressures (cmH_2O) registered during the examination with the intravesical (P1) and the abdominal (P3) manometer, and the detrusor muscle contractile pressure calculated as P1-P3. The urinary bladder was filled with sterile isotonic distilled water at an average rate of 70 ml/min. Sta: start of filling. Fs: time of first sensation of need to void. Pea: peak pressure values during voiding. Sto: pressure values at the time point when voiding was intentionally interrupted by the patient.

Similarly as in the general population (59), the BRS values displayed a significant negative correlation with age (r=-0.44, p=0.012). None of the other test results correlated with age, and none of the above test results correlated with the pSS duration, with the presence of any extraglandular manifestation or immunoserological positivity, with the stimulated saliva production (measured with the Saxon test [74]), or with the results of the sensory nerve function test. When the results of the cardiovascular reflex tests were compared between two subgroups of pSS patients grouped according to whether or not they had symptoms suggestive of a cardiovascular autonomic neuropathy, no differences were found. However, the 9 patients who reported palpitations had lower (i.e. more abnormal) values in all of the HRV and BPV parameters; this difference was statistically significant in the case of rMSSD (p=0.026) and was close to the border of statistical significance in the cases of pNN50 (p= 0.073) and down-BRS (p=0.076). The presence of the other two symptoms did not correlate with any of the results, but the incidence of these symptoms among the patients was very low.

In all of the patients who had symptoms of an autonomic dysfunction and underwent the urodynamic examinations, at least one test result was abnormal. The patient who experienced occasional difficulties in starting voiding was found to have both an abnormally high bladder capacity and decreased peak detrusor muscle contractions. However, the statistical analysis failed to reveal a correlation between the presence of the symptoms and the test results on either the urinary or the gastrointestinal system.

3.4. Preparation of synthetic and recombinant epitopes of human mAchR for testing of their reaction with pSS sera

3.4.1. Patients

Sera were collected from 40 pSS patients (38 female, 2 male, mean age: 55 years, range: 30-82 years; American-European classification criteria [19]), 24 healthy blood donors (mean age: 49 years, range: 23-62 years) and 8 patients with RA (mean age: 56 years, range: 35-79 years).

3.4.2. Recombinant techniques

Complementary DNA (cDNA), coding for the peptide epitope (K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) corresponding to the 2nd extracellular loop of the m4AchR, was chemically synthesised as overlapping 5'-phosphorylated oligonucleotides, which were hybridised together. The synthetic DNA was digested with restriction enzymes and inserted into the expression plasmid pGEX-6P-1, in frame with a sequence coding for glutathione-S-transferase (GST) through a *BamH*I site (monomer form: *GST-M4R)*. In order to construct fusions with multiple blocks of the epitope, the cDNA coding for the second epitope was produced as above, except that the C-terminal amino acid was followed by a Pro-Pro-dipeptide in place of the stop codon and by a *Bgl*II recognition site. The *BamH*I and *Bgl*II cut insert was ligated into the *BamH*I cut plasmid coding for the monomer fusion. As a consequence, the two epitope peptides were separated by a Pro-Pro-Arg-Ser tetrapeptide (dimer form – *GST-M4R-M4R; Figure 8*). The inserts and the adjacent regions in all plasmids were sequenced by the dideoxy chain termination technique. The recombinant fusion proteins were expressed in *E.coli,* and purified by means of affinity chromatography as described in [75].

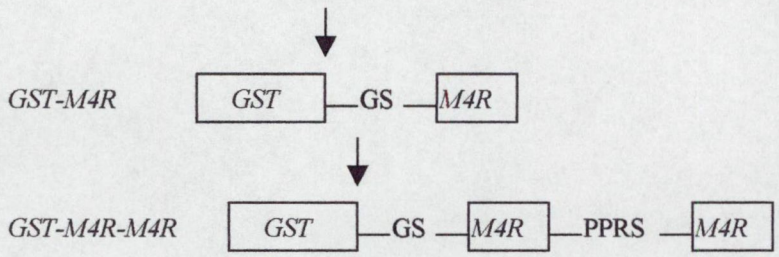


Figure 8. Schematic diagrams of the recombinant products containing the antigenic peptide epitope of the m4AchR.

Each recombinant fusion product was composed of GST at the -NH_2 end, followed by the antigenic epitope(s) at the -COOH terminal. Synthetic DNA sequences were inserted through the *BamH*I site to the GST gene of the fusion expression vector pGEX-6P-1 in frame. A dipeptide of GS (one letter code) was inserted between the GST and the epitope sequence. In the dimer construct, two DNA blocks encoding the peptides were linked to one another in frame by the *BamH*I site without a stop codon, through a sequence coding for a PPRS tetrapeptide sequence. Arrows indicate the fusion sites.

3.4.3. Enzyme-linked immunosorbent assay (ELISA) techniques

The amounts of antigens were calculated by equalising the epitope peptide component of the monomer and the dimer fusion proteins. Likewise, the amount of GST separately used as control for both the monomer and dimer forms was normalised to the GST content of the corresponding fusion. Microtiter plates (Costar, high binding capacity) were coated with 0.9 μg/well GST and 1 μg/well *GST-M4R,* as well as with 0.4 μg/well GST and 0.5 μg/well *GST-M4R-M4R* in 0.1 M Na_2CO_3 buffer, pH 9.6 containing 10 mM dithiotreitol, overnight at 4 °C. The plates were then washed twice with phosphate-buffered saline (PBS), pH 7.4, and the remaining binding sites were blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (blocking solution) for 1 hour at room temperature. This was followed by washing with PBS-Tween (PBS containing 0.05% Tween-20). 100 μl/well of the sera of patients and controls was applied at a dilution of 1/100 in blocking solution and incubated for 2 hours at 37 °C. After washing with PBS-Tween, peroxidase-conjugated antihuman IgG (Sigma) diluted 1:4000 in blocking solution was added to each well (100 μl/well), followed by incubation for 1 hour at 37 °C. After washing, the reaction was developed with *o*-phenylene-diamine in phosphate-citric acid buffer, pH 5.0. The colour was allowed to develop for 15 minutes at room temperature in the dark. The reaction was stopped with 4 N sulfuric acid. The optical density (OD) was measured at 492 nm. Each serum was applied in triplicate. The *(GST-M4R)*-$(GST)_{492\ nm}$ and *(GST-M4R-M4R)*-$(GST)_{492\ nm}$ values for the sera of the patients and the controls were determined as measures of the peptide-specific reaction. Cut-off values were calculated as $x + 2$ SD (where x was the mean OD for the control subjects). Student's *t*-test was used to determine whether the differences between the groups were statistically significant.

3.4.4. Results

As the first step, we chemically synthesised a 25-mer (K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) and a 10-mer (K-R-T-V-P-D-N-Q-C-F) amino acid sequence corresponding to the 2nd extracellular loop of the m4AchR. Use of a peptide-based ELISA system failed to detect a reproducible and well-measurable level of autoantibodies in pSS sera, similarly as reported by Cavill *et al* (62).

We therefore studied the application of carrier-bound peptide epitopes instead of the peptides alone. Scatter plot diagrams of the reactivities of the sera of pSS and RA patients and of healthy controls, determined by ELISA, are shown in *Figure 9*. With the mean + 2 SD of the OD values of the healthy controls taken as the cut-off values, the sera of 2/24 of the healthy controls, 12/40 of the pSS patients and 1/8 of the RA patients gave a positive peptide-specific reaction with the monomer form (*Figure 9.a*), as did the sera of 0/24 of the healthy controls, 28/40 of the pSS patients and 1/8 of the RA patients with the dimer form (*Figure 9.b*). The reactions of the sera of the pSS patients with both recombinant fusion peptides proved to be highly specific (98% and 100%) and sensitive (58.8 and 76.9%) for the monomer and the dimer forms, respectively.

Table VI presents the mean ODs and the standard deviations of the tested sera in the 3 groups of subjects. The pSS sera exhibited significantly higher mean ODs than those of the healthy controls ((*GST-M4R*)-$(GST)_{492\ nm}$: 0.196 *vs* 0.076, $p<0.05$; (*GST-M4R-M4R*) - $(GST)_{492\ nm}$: 0.837 *vs* 0.285, $p<0.0001$) (*Table VI.a*). Significant differences were observed between the pSS and the RA patients as concerns both the monomer ($p=0.0013$) and the dimer peptide ($p=0.0028$) (*Table VI.b*).

Table VI. Results of ELISA assays with two different coating m4AchR-specific antigens.

a.

	Optical density (492 nm)		
ELISA antigens	**pSS**	***p***	**Healthy control**
M4R	0.196±0.039	0.0246	0.076±0.011
M4R-M4R	0.837±0.055	<0.0001	0.285±0.026

b.

	Optical density (492 nm)		
ELISA antigens	**pSS**	***p***	**RA**
M4R	0.196±0.039	0.0013	0.140±0.058
M4R-M4R	0.837±0.055	0.0028	0.436±0.048

Values indicate the mean OD ± SD for all three groups ($n=40$ for pSS, $n=8$ for RA and $n=24$ for healthy control subjects) for the monomer (a) and dimer (b) forms of the antigen. Microplates were coated with 1 μg/well of *GST-M4R* and 0.5 μg/well of *GST-M4R-M4R* antigens, respectively, and incubated with the sera at a dilution of 1:100.

Figure 9. Scatter plot of reactivities of sera of patients with primary Sjögren's syndrome (pSS) or rheumatoid arthritis (RA) and of healthy controls with enzyme-linked immunosorbent assay (ELISA).

A. *GST-MR4* as antigen (1 µg/well); **B.** *GST-MR4-MR4* as antigen (0.5 µg/well). Immobilised fusion constructs were incubated with patient or control serum (diluted 1:100). Binding of autoantibodies to the immobilised antigen was visualised with *o*-phenylenediamine and H_2O_2, after incubation with a peroxidase-labelled antihuman IgG (1:4000). The (*GST-M4R*)-(*GST*)$_{492\ nm}$ and the (*GST-M4R-M4R*)-(*GST*)$_{492\ nm}$ optical densities (ODs) were considered. The cut-off value was set at the OD of the mean of the negative control sera + 2 SD. The cut-off is indicated by the dashed line.

4. Discussion

4.1. Investigation of cardiac and oesophageal involvement in pSS

Our findings indicate that, while overt cardiac disease is rare in pSS, clinically silent changes are common in these patients. Three abnormalities occur in significant proportions of the patients: an echogenic pericardium, a left ventricular diastolic dysfunction and an increased pulmonary arterial pressure. An echogenic pericardium is most probably a consequence of previous symptom-free pericarditis. A precise explanation of the left ventricular dysfunction can not be given; vascular or other causes of an impaired myocardial relaxation are hypothesised. An increased pulmonary arterial pressure is related to a diffuse interstitial lung disease or to direct vascular damage to the pulmonary artery in the involved patients.

In summary, we could identify three types of cardiac abnormalities which seem to belong in the clinical spectrum of pSS, as these abnormalities were detected in high proportions of pSS patients and no other obvious causes could be identified in the affected patients.

4.1.2. Oesophageal dysfunction in pSS

A variety of motor abnormalities were revealed in many pSS patients, with a decreased oesophageal body peristaltic velocity being the most common (44% of the patients) and most significant phenomenon. We concluded that both a diminished saliva production and an impaired oesophageal body peristaltic activity are factors in the development of swallowing difficulties in pSS. We hypothesised the role of a parasympathetic dysfunction in the elicitation of the oesophageal motor abnormalities.

4.2. Impaired microvascular response to cholinergic stimuli in pSS patients

In this study, we examined the cutaneous cholinergic vasodilation in pSS patients in response to the local administration of a muscarinic cholinergic agonist, carbachol. Acetylcholine had been demonstrated to cause vasodilation in healthy humans (64).

Carbachol had been demonstrated to induce phospholipase C-dependent saliva secretion through stimulation of the muscarinic cholinergic receptors in human salivary acinar cells [76,77]. The same muscarinic receptors had been implicated in the cholinergic vasodilation in human cutaneous arterioles in response to body heat stress [64]. These findings provided a basis for our choice of an investigation of the cutaneous microcirculation as a model of the parasympathetic innervation of the salivary glands.

We found that the average increase in cutaneous microcirculatory blood flow in response to carbachol was significantly smaller in the pSS patients than in the healthy controls. In half of the pSS patients, the reaction to a potent parasympathomimetic drug was virtually absent or markedly diminished.

This study was among the first involving *in vivo* examinations of the pathophysiology of the autonomic innervatory pathway in pSS patients. Since a muscarinic receptor agonist was administered directly to the examined target organ, the detected unresponsiveness was strongly suggestive of a dysfunction at a receptorial or postreceptorial level. On the basis of these speculations, and supported by contemporary publications [58,59], we hypothesised that a likely explanation of a parasympathetic dysfunction in pSS is the interaction of anti-mAchR antibodies with the acetylcholine-receptor. More recent investigations seem to lend support to our hypothesis [60,78,79].

It has been hypothesised that an interaction may occur in the salivary glands between the infiltrating inflammatory cells and certain neuropeptides (55). *Vice versa*, another hypothesis suggests that the loss of trophic stimuli from the parasympathetic nerves in the salivary glands might be a cause of acinar cell atrophy, and this may contribute to the decreased saliva production (55). Our results relating to the investigation of a target organ distant from the areas of lymphocytic inflammation suggest that a disorder of the cholinergic receptors may not be restricted to the exocrine glands, but may be a more widely occurring phenomenon, and that this defect is possibly independent of the local inflammatory process.

4.3. Clinical examinations of an autonomic nervous system dysfunction

The methods applied in this clinical trial were accepted methods for the assessment of autonomic neuropathy; however, as anti-AchR autoantibodies are a special feature in pSS, and these antibodies may also influence the innervation mechanism, we preferred use of the term autonomic dysfunction to autonomic neuropathy.

As already mentioned in the introduction, the studies of a cardiovascular autonomic neuropathy had yielded inconsistent results, in part possibly because of the different methods applied. Therefore, we investigated the cardiovascular autonomic dysfunction with both of the most widely used methods. The examination of HRV and BPV revealed that, while having a normal HR and BP in general, a great majority of the pSS patients have a restricted variability of both HR and BP. Our results conclusively indicated that signs of both a sympathetic and a parasympathetic cardiovascular autonomic dysfunction can be observed in pSS patients.

The cardiovascular reflex tests also revealed marked differences as compared with the healthy controls, but these data were far less consistent. The ratio of individuals with an abnormal result was higher among the patients than among the controls in all 5 tests, but in only 2 of the 3 parasympathetic tests and in 1 of the 2 sympathetic tests was this difference statistically significant. The autonomic neuropathy score was abnormal in 85% of the pSS patients. However, the median global score was 3, which is a borderline value, being much lower than that in diabetic patients, in whom average scores of 6 or even higher are the typical published values [80,81].

It is widely accepted that autonomic reflex tests perform best in diabetic populations [72]. Therefore, these tests are well suited for the mass screening and risk stratification of diabetics in routine clinical practice [82]. More subtle abnormalities of the cardiovascular autonomic regulation might be difficult to assess by reflex test methods. These considerations probably explain why we failed to obtain consistent results with the cardiovascular reflex tests on pSS patients. Another potential explanation why the previous studies failed to lead to conclusive results may be the method of control selection. Age- and sex-matched healthy volunteers were recruited, and the control/patient ratio seldom exceeded 1:1. However, some of the variables assessed in the present study, such as the spontaneous BRS, had been shown to vary tremendously in the healthy population [71,83]. Comparisons with single age- and

sex-matched control subjects may therefore be subject to chance influences. Accordingly, we related the variables of the pSS subjects to a large database on age- and sex-matched healthy volunteers.

A conclusion of the investigations on the cardiovascular autonomic dysfunction is that it is common, but clinically usually relatively mild in pSS. Our patients rarely experienced symptoms potentially attributable to it, and significant cardiac symptomatology has not been described in studies on large cohorts of pSS patients [11]. Nevertheless, upon direct questioning, 9 of the pSS patients (18%) reported episodes of palpitations. Notably, in these patients, the autonomic dysfunction was more pronounced than in the pSS patients without this symptom when examined with HRV/BPV measurements, while the reflex tests failed to identify these patients. We concluded that it is not warranted to routinely test every pSS patient, but in occasional pSS patients with complaints suggestive of an autonomic dysfunction, short-term HRV/BPV examinations are useful for the verification of an underlying autonomic nervous system abnormality, while the cardiovascular reflex tests are less valuable in this respect, because of their lower sensitivity.

In patients with diabetes mellitus, examination of the gastric emptying is an accepted method of indication of gastrointestinal autonomic neuropathy [81]. The parasympathetic influence predominates in the regulation of the upper gastrointestinal tract motility, including the stomach [84]. A loss of vagal tone, resulting in the inhibition of gastric emptying, is characteristic of an autonomic neuropathy in diabetes mellitus [85]. By means of gastric emptying scintigraphy, severely delayed gastric emptying was observed in many diabetic patients [81,86]. Our results indicated that the gastric emptying is prolonged in 70% of pSS patients, and a considerable proportion of these patients exhibit a markedly decreased gastric motility. As organic changes that may influence the gastric emptying were not detected in the patients, this abnormality is most probably a consequence of an impaired parasympathetic activity. Data on gastric emptying in pSS patients have been reported in only one study, aimed at an evaluation of the oesophageal involvement in scleroderma and pSS (57). Similarly to ourselves, the authors found that the gastric emptying was slower in the pSS patients than in the controls. It is noteworthy that pSS patients rarely had complaints of an impaired gastric emptying, and even the 8 patients in our study who experienced postprandial fullness merely had mild symptoms which they reported only upon direct questioning.

In our above-mentioned investigations of the oesophageal motor function in pSS patients, the predominant abnormality appeared to be a decreased oesophageal body peristaltic velocity. As the oesophageal body muscles are partly innervated via the parasympathetic nervous system, we suggested a cholinergic autonomic dysfunction as one potential explanation of an impaired oesophageal motor function. Those findings are in keeping with the results of this study, and suggest that abnormalities in the upper gastrointestinal tract attributable to a parasympathetic autonomic dysfunction can be detected in a great proportion of pSS patients. However, we note that we failed to reveal a correlation between the abnormalities of the gastric and the oesophageal motility measured in 6 patients who participated in both trials.

This was the first report on a systematic search for signs of an autonomic dysfunction involving the urinary bladder in pSS patients, despite some reports of urinary retention in patients with this disease. However, animal experiments had demonstrated that serum from pSS patients has an inhibitory effect on isolated rabbit urinary bladder smooth muscle contractions, this effect being mediated by anti-m3AchR antibodies [78]. The normal function of the urinary bladder is mainly under the control of the parasympathetic nervous system, which facilitates the contraction of the detrusor muscle and the relaxation of the internal sphincter. The loss of parasympathetic stimuli due to sacral plexus injury or diabetic autonomic neuropathy leads to urinary retention, bladder atonia or flaccidity [87]. The difficulty in starting voiding mentioned by one patient may be explained by a parasympathetic dysfunction, for an obstructive disorder had been excluded. Similarly to the results on the gastrointestinal system, asymptomatic changes, i.e. an increased bladder capacity, a decreased detrusor pressure and a decreased maximal uroflow, were detected in approximately half of the patients. As obstructive changes or other organic pelvic diseases were excluded, these results are consistent with a decreased detrusor muscle tone and contractility, and, similarly as with flaccid neurogenic bladder, a decreased parasympathetic influence can be suspected [87].

Finally, we attempted to find an explanation to the finding of the high prevalences of the signs of an autonomic dysfunction in all of the examined organs. In view of some marked differences from diabetic patients, in whom an autonomic dysfunction is predominantly mediated by an autonomic neuropathy, we hypothesised that other factors might also play a role in the elicitation of an autonomic failure in pSS patients. A further potential explanation

of an autonomic dysfunction is the interference of antireceptor antibodies with the normal innervation process. Similarly as for the salivary and the lachrymal glands, the functionally predominant muscarinic receptor subtype in the gastrointestinal and urinary tracts is the m3AchR subtype [66,67], while the heart contains the m2AchR subtype [88]. As the clinical pattern of the autonomic dysfunction in the various organs could be explained by the presence of anti-mAchR antibodies of different receptor subtype specificities, and some experimental data also raised the possibility of antireceptor antibody-visceral organ interactions [78,79], we concluded that it is worthwhile to examine whether anti-mAchR antibodies with different antigenic specificities may be detected in pSS patients with different clinical patterns of autonomic dysfunction.

4.4. Antimuscarinic-receptor antibody assays

As already mentioned in the Introduction, the results of the two previous published studies involving immunoassays of pSS sera and human mAchR-peptide sequences yielded contradictory results. Therefore, we attempted to develop an ELISA assay with a different mode of antigen preparation, i.e., with the use of recombinant fusion proteins containing the human m4AchR epitope. Nevertheless, in our preliminary experiments, we also produced a synthetic m4AchR-specific peptide and its shorter variant, and, similarly to Cavill *et al* [62], we too failed to detect a reliable immunological reaction *(data not shown)*. With regard to previous work in the field of peptide-coating techniques, we speculated that the physicochemical properties of the peptide used as an immunodominant epitope of the human m4AchR (a hydrophobic character and a tendency to form dimers through its two cysteine residues [89]) can influence its binding capacity to the microplate and also its antigenicity.

Subsequently, to avoid the rather problematic peptide-based ELISA system, we applied the above-mentioned antigenic peptide with a carrier protein of GST. We did this for three reasons: 1. To establish a more standard amount of the coated mass of the antigen, which is an important factor in ELISA reactivity. 2. To achieve a better orientation and/or conformation of the peptide epitope with the help of the GST moiety. 3. To increase the affinity of the antigen for the antibody, since our recombinant strategy permits the construction of fusions with dimeric epitope peptides.

We have demonstrated that the ELISA investigations with the fusion forms of the antigenic peptide are reliable and reproducible. Moreover, use of the dimeric epitope fusion as substrate leads to an increase in the sensitivity of the procedure as compared with the use of an equal amount of peptide in a single, non-repeated form. With reference to our biochemical studies on this topic [90], we consider that the higher efficiency and fidelity of the GST-homodimer fusion product can be explained by the avidity effect of GST dimer formation and a better ligand affinity due to the beneficial conformational consequences provided by the protein microenvironment. With optimum adjustment of the procedure parameters, we attained a specificity of 100% and a sensitivity of 76.9% for this assay. Although this sensitivity is lower than that reported by Bacman *et al* (61), we believe that the elimination of the above-mentioned disadvantages of the peptide ELISA method makes our results reproducible. We also expect that a more specific characterisation of the antigenic epitope in future studies with the m3AchR will probably lead to assays with improved sensitivity as compared with the present one.

5. *Summary of the results presented in the thesis*

- *ad 1:* As a co-investigator, I participated in studies which described two previously unrecognised features of pSS: an impaired left ventricular diastolic function and an echogenic pericardium. After conflicting results in this topic, oesophageal manometric studies revealed an impaired oesophageal body peristaltic velocity as the major motility disorder in a majority of the patients.
- *ad 2:* In one of the first human, functional, *in vivo* studies, I demonstrated an impaired responsiveness of pSS patients to cholinergic stimuli. These results were among the first proofs of a parasympathetic dysfunction in pSS. This was the first experimental model to prove directly that this unresponsiveness is a general feature in pSS (i.e. it is not confined to the exocrine glands), and that it is based upon a functional disturbance at a receptorial level.
- *ad 3:* With a combination of both previously applied methods, I provided data that seem to clarify the occurrence, severity and clinical correlates of a cardiovascular autonomic nervous system dysfunction in pSS patients. With the use of human clinical tests, I gave the first description of the widespread occurrence of disturbances in the urinary parasympathetic innervation, and I was the second to demonstrate this phenomenon in the gastrointestinal tract.
- *ad 4:* We developed an ELISA system for the detection of recombinant human antimuscarinic-receptor antibodies in pSS patients. After previous publications of unsuccessful trials in this field, we were able to prove that a majority of pSS patients have autoantibodies reacting with human mAchR-specific epitopes, and this test identifies pSS patients with considerable sensitivity and specificity. On the basis of comparative studies with synthetic and recombinant fusion peptides, we explored certain physico-chemical and antigenic characteristics of mAchR peptides that may aid in the future development of diagnostic immunoassays for anti-mAchR antibodies.

6. References

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7. List of abbreviations

ANA:	antinuclear antibody
anti-SSA:	anti-Sjögren's syndrome A antibody
anti-SSB:	anti-Sjögren's syndrome B antibody
BP:	blood pressure
BPV:	blood pressure variability
BRS:	baroreflex sensitivity
CD4:	cluster of differentiation 4
cDNA:	complementary DNA
CVR:	cutaneous vascular resistance
DBP:	diastolic blood pressure
dCVR:	delta-cutaneous vascular resistance (change in the CVR)
dSBF:	delta-skin blood flow (change in the SBF)
ECG:	electrocardiogram
ELISA:	enzyme-linked immunosorbent assay
GST:	glutathione-S-transferase
HF-RRI:	high-frequency peak of the variability in the R-R wave intervals
HF-SBP:	high-frequency peak of the variability in the systolic blood pressure values
HLA:	human leukocyte antigen
HR:	heart rate
HRV:	heart rate variability

IgG:	immunoglobulin-G
LF-RRI:	low-frequency peak of the variability in the R-R wave intervals
LF-SBP:	low-frequency peak in the variability in the systolic blood pressure values
mAchR:	muscarinic acetylcholine receptor
MALT:	mucosa-associated lymphoid tissue
MBP:	mean arterial pressure
MRI:	magnetic resonance imaging
NOD:	non-obese diabetic (mouse)
OD:	optical density
PBS:	phosphate-buffered saline
PKC:	protein kinase C
pNN50:	the percentage of R-R wave intervals that differed from the adjacent interval by > 50 msec
pSS:	primary Sjögren's syndrome
RA:	rheumatoid arthritis
RF:	rheumatoid factor
rMSSD:	square root of the average of the squared differences between each consecutive pair of R-R wave intervals
RRI:	R-R wave interval
SBF:	skin blood flow
SBP:	systolic blood pressure
SD:	standard deviation
SDRR:	standard deviation of the mean R-R wave interval
SS:	Sjögren's syndrome
$t_{1/2}$:	(emptying) half-time

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